Vascular cell adhesion molecule-1 up-regulation and phenotypic modulation of vascular smooth muscle cells predate mononuclear infiltration in transplant arteriopathy

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Objective: Expression of embryonic myosin heavy chain isoforms and vascular cell adhesion molecule-1 by neointimal vascular smooth muscle cells are independent indicators of atherosclerotic plaque development in both human beings and experimental animal models. We examined the chronologic change in smooth muscle cell myosin heavy chain isoforms, vascular cell adhesion molecule-1 expression, and mononuclear cell infiltration in a carotid arterial transplant model to ascertain whether similar phenotypic changes would occur in transplant arteriopathy.

Methods: Transplanted rabbit carotid arteries were examined at 7, 14, 21, and 35 days (n = 5, 7, 6, and 5, respectively). Lesion progression and the prevalence of smooth muscle cell myosin heavy chain isoforms, T-lymphocytes, macrophages, and vascular cell adhesion molecule-1 expression were evaluated immunohistochemically by computerized image analysis.

Results: In this carotid arterial transplant model, the intima/media area ratio increased significantly by 35 days ($P = .01$) as cell density decreased ($P = .01$), suggesting extracellular matrix elaboration. Intimal smooth muscle cells expressing embryonic phenotypes were seen as early as 7 days, a phenotypic change that predated mononuclear cell infiltration of the graft by at least 7 days. By 35 days, up to 70% of intimal smooth muscle cells expressed the embryonic phenotype, coinciding with the transition from inflammatory to chronic lesions. Although, in early lesions, vascular cell adhesion molecule-1 was identified on luminal endothelium overlying mononuclear infiltrates, in advanced lesions vascular cell adhesion molecule-1 was identified primarily on intimal vascular smooth muscle cells.

Conclusions: Overall, these vascular smooth muscle cell changes mark important early events in transplant arteriopathy that may not be ameliorated by immunosuppressive regimens in routine use.

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Post-transplant coronary arteriopathy remains the major unsolved problem limiting long-term survival after heart transplantation. Current routine post-transplant immunosuppression, directed at host alloimmune reactivity, may offer little to reduce the smooth muscle cell (SMC) proliferation that characterizes post-transplant arteriopathy. Understanding the transformation that vascular SMCs undergo during the development of these proliferative intimal lesions is necessary for rational drug

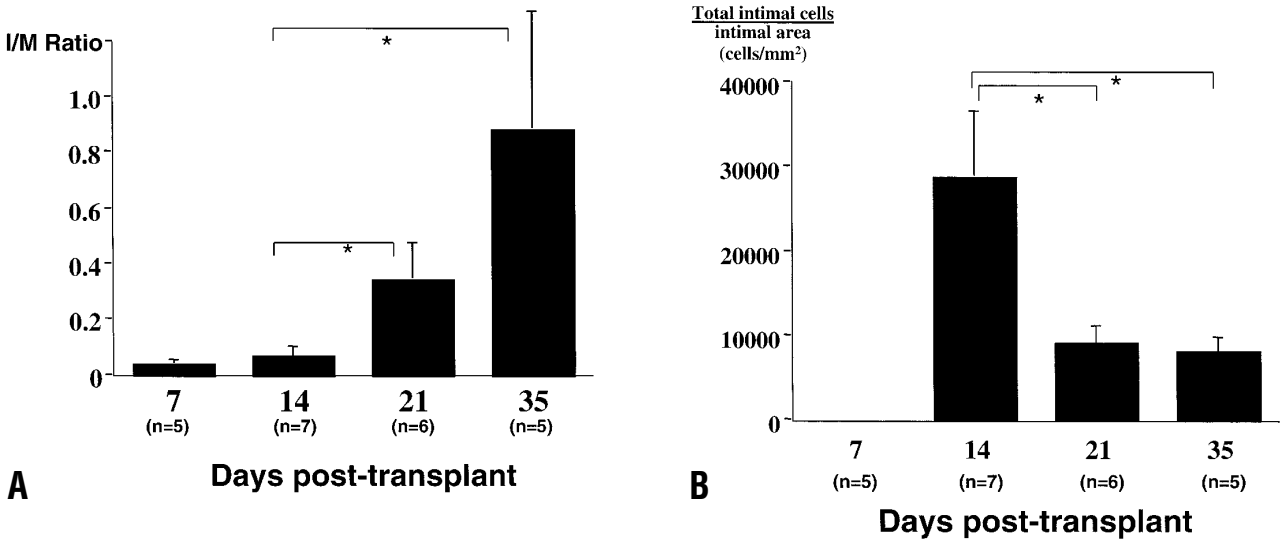

Figure 1. A, Intimal lesion development (intima/media area ratio). The intima/media *(I/M)* area ratio was significantly different between specimens at 14 days and those at later time points. *Error bars* denote the standard deviations. *Multiple comparison *P* = .01 versus 14 days. B, Cell density was measured as the total number of intimal cells divided by the total intimal area. There was a significant decrease in cell density between specimens at 14 days and those at later time points, suggesting extracellular matrix production at the later time points. *Error bars* denote the standard deviations. *Multiple comparison *P* = .01 versus 14 days.

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development for this lethal problem. In nontransplant atherosclerosis, expression of vascular cell adhesion molecule (VCAM-1) on endothelium and on neointimal SMCs is related to plaque development both in rabbit models[1,2] and in human coronary disease.[3,4] We hypothesized that VCAM-1, as an adhesion molecule specific for mononuclear cells, should play an equally important role in the developing lesions of transplant arteriopathy, theorizing that immune arteriopathy would be regulated through mononuclear cells. We tested this hypothesis in a rabbit model of carotid arterial allotransplantation.

In addition to VCAM-1 expression, the appearance of an embryonic or fetal myosin heavy chain isoform has been reported to be a molecular marker specific for neointimal lesion development in nontransplant atherosclerosis.[5-8] Among the myosin heavy chain isoforms, the SM1 isoform is the universally present phenotype, present at all developmental stages, and SM2 is the common adult phenotype, seen only postnatally in normal vascular SMCs.[9,10] Both phenotypes are alternative RNA splicing products that originate from a single gene, regulated developmentally.[5,6] An embryonic isoform, SMemb, or nonmuscle myosin heavy chain B, is a fetal or embryonic phenotype that has been found, to date, in human beings predominantly in fetal life, and, in adults, in ductus arteriosus remnants and, pertinently, in the neointimal lesions of nontransplant atherosclerotic plaques.[8,11] All of these human myosin heavy chain isoforms share more than 90% homology with their counterparts in the rabbit.[8] In rabbit tissue specimens, similar heterogeneity in myosin heavy chain isoforms has been reported under designated pathologic conditions in vivo, such as restenosis after balloon injury[12-14] and in diet-induced atheromata.[7,15-17] In nontransplant arterial disease, such phenotypic alterations appear to herald neointimal formation.[12,16-18] These experiments investigate whether similar phenotypic modulation in SMCs would occur in transplant arteriopathy and whether such a change would have the same implications for disease progression.

Methods

Animals and Experimental Procedures

SMC transformation was investigated in a new rabbit model of transplant arteriopathy, an orthotopic carotid arterial transplant model between two outbred strains of rabbits: New Zealand White donors and Stauffland recipient rabbits. Carotid arterial grafts were used rather than venous interposition grafts to avoid the disparities in vascular wall thickness, distensibility, and myosin heavy chain composition between arteries and veins.[14,19] Rabbits were used because their arterial disease closely replicates human disease[20,21] and because immune recognition sites in the rabbit have close parallels to the human histocompatibility loci.[22] No immunosuppression was used to eliminate confounding pharmacologic variables.

Donor New Zealand White rabbits (mean weight 1.9 kg) were anesthetized with a mixture of ketamine (35 mg/kg), xylazine (7 mg/kg), and atropine (0.05 mg/kg), given intramuscularly, and then intubated and mechanically ventilated with

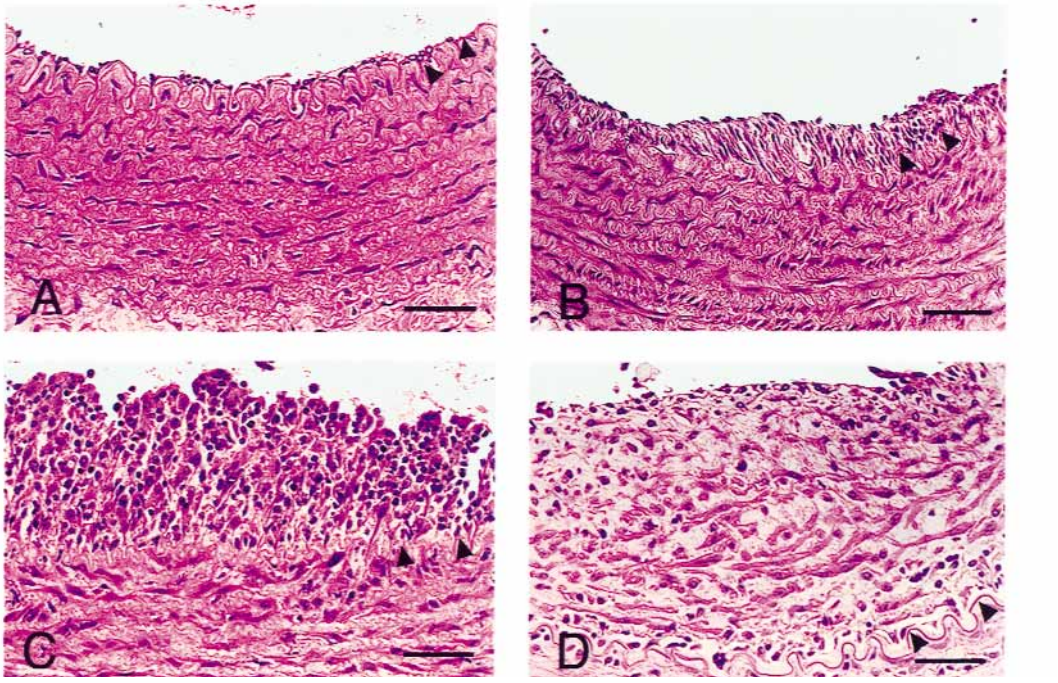

Figure 2. Lesion progression in transplanted rabbit carotid arteries: Selected representative specimens at different time points after transplantation. The *arrowheads* mark the internal elastic lamina in all specimens. (Hematoxylin/eosin stain; *magnification bar* = 50 μm.) A, Seven days: Minimal lesion is present; single cells are seen above the internal elastic lamina. B, Fourteen days: The lesion is now several layers deep and a few mononuclear cells are seen. C, Twenty-one days: An extensive mononuclear infiltrate is evident, with scattered mononuclear cells in the media. D, Thirty-five days: An example of a late specimen in which the character of the lesion has changed toward a chronic appearance. A preponderance of SMCs with large nuclei and spindle-shaped cytoplasm is seen. The density of cells is notably less, suggesting extracellular matrix production.

100% oxygen and halothane. After systemic heparinization (500 U/kg), both carotid arteries were harvested from the donor rabbit, followed by euthanasia with pentobarbital infusion. Each donor rabbit provided grafts for two recipient rabbits. Donor carotid grafts were immersed in cold Ringer's lactate solution until implantation. Recipient Stauffland rabbits (mean weight 1.8 kg) were similarly anesthetized and the left carotid arteries surgically exposed. After intravenous heparinization (250 U/kg), the common carotid artery was isolated between two atraumatic vascular clamps and excised. The harvested donor carotid arterial graft was interposed orthotopically between the divided ends of the recipient carotid, with interrupted 8-0 Prolene sutures (Ethicon, Inc, Somerville, NJ) being used for the anastomoses. Graft warm ischemic time was approximately 20 minutes for the implantation procedure, although total (warm plus cold) ischemic times for the grafts varied between 30 minutes and 70 minutes because the two recipient operations using the two carotids from a single donor were performed sequentially. Pairs of recipient rabbits, one with a shorter and one a longer ischemic time, were randomized to different protocol groups to ensure equal representation of shorter and longer ischemic times among the four groups.

Recipient rabbits were anesthetized, heparinized, and euthanized for carotid excision on postoperative days 7 (group 1, n = 5), 14 (group 2, n = 7), 21 (group 3, n = 6), and 35 (group 4, n = 5). In each rabbit, both the transplanted left carotid artery and the untransplanted native right carotid artery were harvested. The untransplanted contralateral native carotid artery was used to provide an internal control since these native carotid arteries in the transplant recipient animal were exposed to the same circulating cytokines as the transplanted carotid arteries.

Another set of rabbits underwent sham operations to provide further controls for the effects of surgical manipulation and ischemia without transplantation. Sham-operated carotids were harvested at the same time points (n = 2 at 7, 14, 21, and 35 days). In the sham operations, the recipient carotid artery was similarly dissected free from connective tissue and clamped for an ischemic time equal to the average ischemic time of transplanted grafts. During the ischemic time, sham-operated arteries were nearly totally transected and then resutured (essentially an autotransplant) to expose these carotids to the same surgical trauma and ischemic times as the transplanted carotid arteries.

All experiments were performed in accordance with the "Principles of Laboratory Animal Care," formulated by the National Society for Medical Research, and the "Guide for the Care and Use of Laboratory Animals," prepared by the Institute of Laboratory Animal Resources and published by the National Research Council (National Academy of Sciences, revised 1996).

Tissue Preparation

Carotid artery specimens were sectioned transversely and fixed immediately. Zinc fixation buffer was used to detect antibody Rb1/9 to VCAM-1, because this fixative allows discrimination of cellular morphologic features not possible in frozen sections.[23] For other immunocytochemical studies, specimens were fixed in methyl Carnoy's solution (methanol/chloroform/glacial acetic acid = 60:30:10). All specimens were dehydrated through graded methanol concentrations and embedded in paraffin. In each block, two sections at the center of each graft were stained with standard hematoxylin and eosin and adjacent serial sections were prepared for immunocytochemical evaluation.

Immunohistochemistry Methods

Serial 6-μm cross-sections of paraffin-embedded tissue were used for immunocytochemistry to identify T-lymphocytes, macrophages, and expression of both VCAM-1 and vascular SMC myosin heavy chain isoforms (SM1, SM2, SMemb). Antibodies included monoclonal antibody (mAb) TIB 188 (American Type Culture Collection, Rockville, Md) for rabbit T-lymphocytes, mAb RAM-11 (Dako Co, Carpenteria, Calif) specific for rabbit macrophages, mAb Rb1/9 (a generous gift from Dr Myron Cybusky), specific for rabbit VCAM-1,[1] and antibodies to SM1, SM2, and SMemb myosin heavy chain isoforms[5] (generous gifts from Dr Ryozo Nagai).

To control for endogenous peroxidase activity, we preincubated sections with methanol/peroxide (1:1) and then transferred them to phosphate-buffered saline solution with 0.5% bovine serum albumin for 30 minutes. So that VCAM-1 expression could be visualized, sections were incubated overnight at 4°C in primary antibody to rabbit VCAM-1 (mAb Rb1/9). For other antibodies, exposure to primary antibody was conducted at room temperature for 60 minutes. Sections were then incubated in biotinylated horse anti-mouse immunoglobulin for 30 minutes and signal-amplified with the ABC Elite avidin-biotin peroxidase technique (Vector Laboratories, Burlingame, Calif). The black reaction product was developed by means of 3,3´ diaminobenzidine with nickel chloride. Slides were counterstained with methyl green to label nuclei, then dehydrated and mounted.

Assessment of the Intimal Lesion

For all assessments, including both intimal lesion calculations and cell type composition, slides were read in a blinded fashion and analyses were performed in duplicate as a check on the accuracy of the results.

The extent of intimal lesion development was quantified by calculating the intima/media area ratio obtained from tracing the perimeter margins of the intima and media on computerized image analysis (Optimus, Bothell, Wash). This ratio allows comparisons to be made between arteries of different diameters, thereby reducing both individual variations between rabbits and the potential for vascular spasm as confounding variables.

Intimal cell density was calculated as the total number of intimal cells divided by the intimal area. The total number of intimal cells was obtained by counting the number of intimal nuclei using the same software on specimens stained with hematoxylin and eosin.

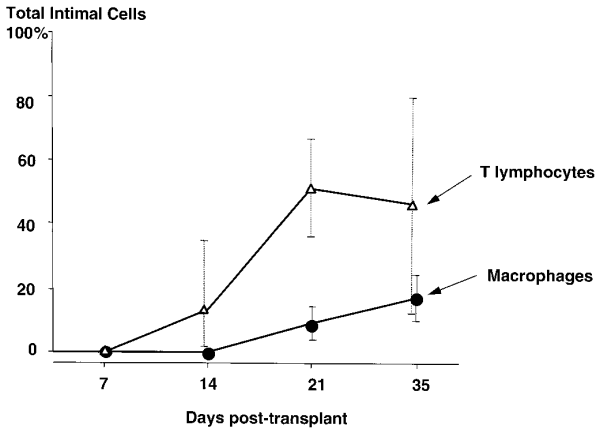

Figure 3. Time course of T-lymphocyte and macrophage infiltration into the intima. In these specimens, T-lymphocytes were first seen at 14 days, and macrophages appeared by 21 days. *Error bars* denote the standard deviations of the means.

Quantitation of Myosin Isoforms, VCAM-1 Expression, and Mononuclear Infiltrates

On immunohistochemistry, the areas within the intima and media staining positively with antibodies specific for rabbit VCAM-1, T-lymphocytes, macrophages, and the three SMC heavy chain isoforms were defined by computerized image analysis. Mouse immunoglobulin was used as the control for nonspecific antibody binding on serial sections run in parallel. Appropriate adjustment of the gating threshold was necessary to control for variations in staining intensity between specimens. In some specimens, stained cells were counted manually on an RGB image monitor at ×400 magnification. For the myosin heavy chain isoforms SM2 and SMemb, the immunoreactive area defined by SM1 staining was used as the denominator to determine the ratio of each phenotype compared with the total SMC (SM1-expressing) area.

Statistical Methods

Group mean values were compared by 1-way analysis of variance with P values adjusted for multiple comparisons by the Student-Newman-Keuls method. When necessary, a log transformation was applied to the data to stabilize variances. All statistical analyses were done on a personal computer with SPSS for Windows version 9 statistical software (SPSS, Inc, Chicago, Ill).

Results

Lesion Progression in this Model of Orthotopic Carotid Allograft Arteriopathy

Intima/media area ratio. Intimal lesion development accelerated with time (Figure 1, *A*). In our preliminary work in this model, an intimal lesion developed in transplanted carotid arteries of untreated recipient rabbits by 21 days. In this study, we extended the time points to examine specimens out to 35 days as well to document the transition into a chronic lesion that closely resembles proliferative human transplant arteriopathy (Figure 2). There was a significant

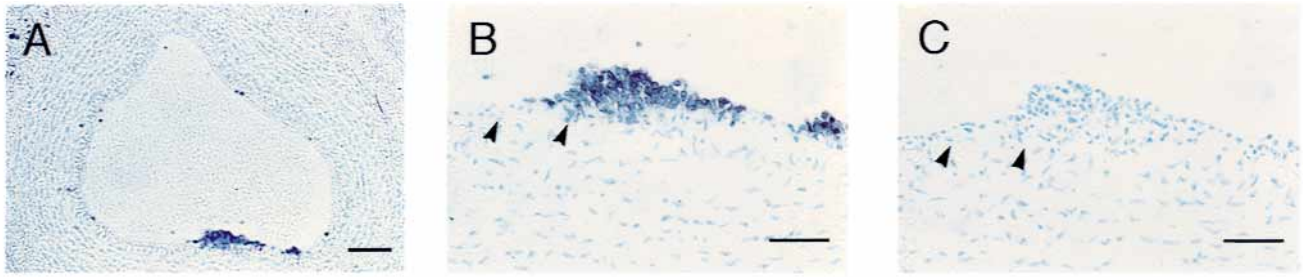

Figure 4. Early appearance of mononuclear cells in a 14-day specimen. On all immunocytochemistry specimens, specific antibodies are visualized by a black reaction product. (Methyl green nuclear counterstain; *arrowheads* mark the internal elastic lamina in panels B and C.) A, T-lymphocytes: Although a circumferential lesion has developed, T-lymphocyte infiltration *(in black)* is still infrequent and localized (mAb TIB 188; magnification bar = 100 μm). B, T-lymphocytes at higher magnification confined to the intima above the internal elastic lamina (mAb TIB 188, *magnification bar* = 50 μm). C, Macrophages: No macrophages are present at 14 days (mAb Ram-11, *magnification bar* = 50 μm).

difference in the intima/media area ratio between specimens at 14 days and those at the two later time points (multiple comparison $P = .01$).

Cell density. A significant decrease in cell density was observed between 14 days and later time points (multiple comparison $P = .01$), suggesting a substantial increase in extracellular matrix production (Figure 1, *B*). This change in cell density coincided with the transition from an inflammatory to a chronic, SMC-based lesion (Figure 2, *D*).

Mononuclear cell infiltration into the intima. T-lymphocytes were not detected in either the intima or the media on the 7-day specimens (Figure 3). At 14 days, occasional and very localized collections of T-lymphocytes appeared in the intima (Figure 4), but medial T-lymphocytes were still rare. By 21 days, T-lymphocytes had become the major component of the developing lesion. The appearance of macrophages in the lesions lagged behind the T-lymphocytes, being first detected at 21 days and not becoming abundant in the developing lesion until 35 days.

Intimal SMCs. SMCs, detected as cells expressing the SM1 isoform, could be seen in the intima as early as 7 days in all transplanted grafts. The total number of intimal SMCs increased with time but accelerated rapidly between 14 and 35 days, coinciding with the change in cellular composition (Figure 1, Table 1).

Phenotypic Switch in SMC Heavy Chain Myosin Isoforms With Neointimal Formation

Over the 35 days, phenotypic modulation occurred in the myosin heavy chain isoforms in the SMCs comprising the intimal lesion (Figure 5). The embryonic phenotype, SMemb, which was not present in vascular SMCs in normal arteries, could be seen in intimal SMCs as well as in medial SMCs located just below the internal elastic lamina as early as 7 days (Figure 5, *C*). The percentage of SMCs co-expressing SMemb increased between 14 and 21 days until well over half (70%) of the neointimal SMCs at 35 days were of the embryonic phenotype (Figure 5, *I,* Table 1).

VCAM-1 Expression in Carotid Allografts

Early phase. VCAM-1 expression was occasionally seen on luminal endothelial cells at 7 days (Figure 6, *A*). Interestingly, in the 14-day specimens, endothelial VCAM-1 expression was often found overlying the area of T-lymphocyte infiltration, suggesting that VCAM-1 may be directly responsible for lymphocyte recruitment into the lesion at this time point (Figure 6, *C* and *D*).

Late phase. By the two later time points, at days 21 and 35, VCAM-1 was identified not only on endothelial cells but also on intimal SMCs, where its expression appeared to co-localize with intimal SMC expression of the embryonic myosin heavy chain phenotype on histologic sections (Figure 6, *E* to *H*).

Sham-Operated Controls

In contrast to the transplanted carotids, there was no significant intimal lesion and no mononuclear infiltration in the sham-operated arteries at 35 days nor in the unoperated native control arteries (Figure 7). The embryonic SMC phenotype, SMemb, was not seen in either the 7-day or 35-day sham-operated grafts. Also, VCAM-1 was not seen, even on endothelium, in either the 7-day or the 35-day sham-operated grafts. These sham controls demonstrate that surgical manipulation and a relatively short ischemic time does not, by itself, cause SMC infiltration, dedifferentiation, or VCAM-1 up-regulation.

Effects of Graft Ischemia

The effect of carotid graft ischemic time (30 minutes vs 70 minutes) on intimal lesion formation was analyzed separately. There was no difference in the extent of lesion or mononuclear infiltration between the grafts with short versus longer total ischemic times (data not shown).

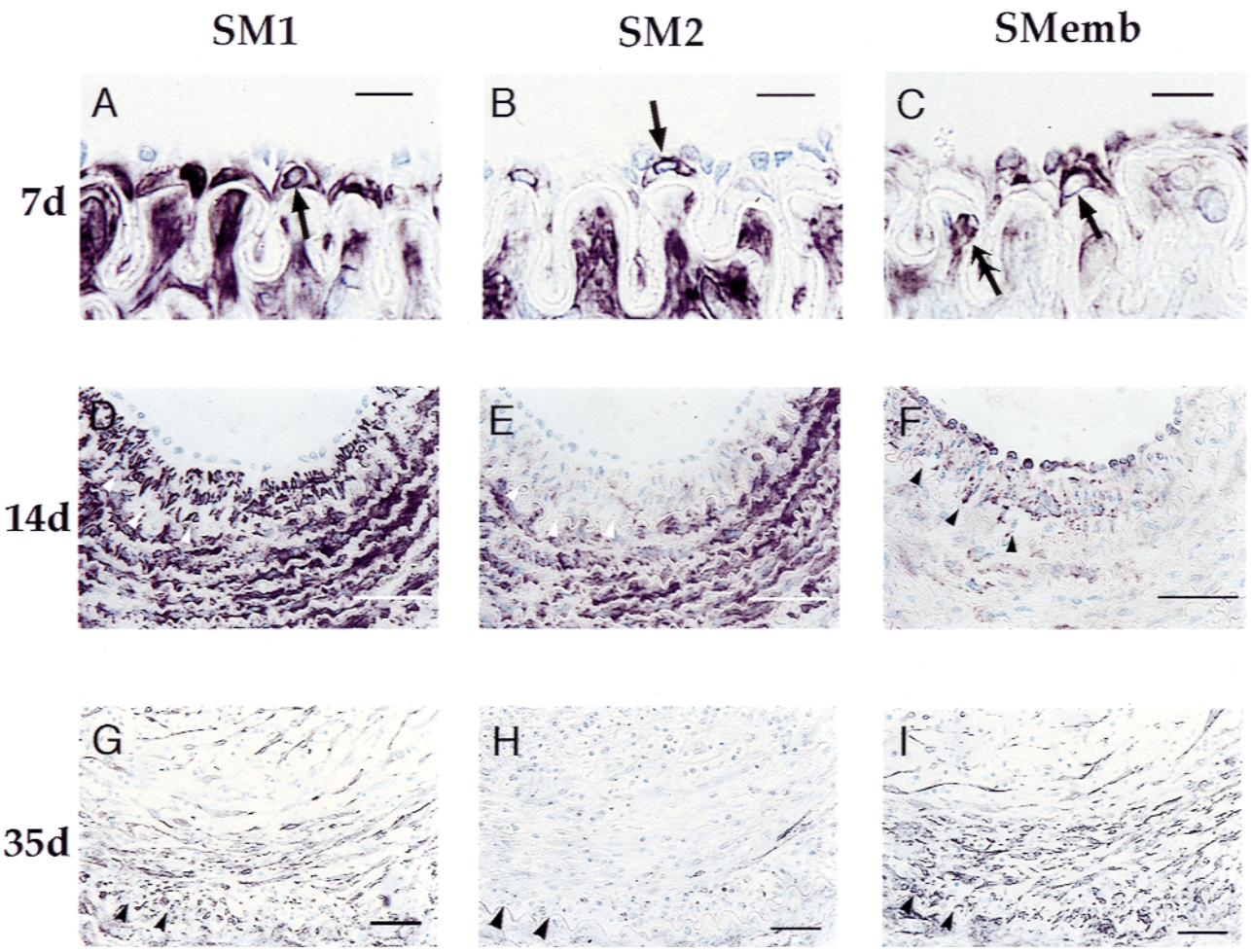

Figure 5. SMC phenotypes in developing intimal lesions. (Methyl green nuclear counterstain; *white* and *black arrowheads* both mark the internal elastic lamina in different panels.) In each column, cells staining positively with antibody to SM1, SM2, or Smemb on these immunocytochemistry panels are visualized as black. A to C, Seven-day specimens: A, mAb to SM1; B, SM2; C, SMemb (embryonic phenotype). A, Although only a few intimal cells are seen at this early stage, SMCs are already detectable in the intima above the internal elastic lamina *(arrow).* All intimal SMCs express SM1. B, Some of these early intimal SMCs also express the SM2 phenotype *(arrow).* C, The embryonic phenotype, SMemb, is expressed by intimal SMCs *(arrow)* as early as 7 days as well as by endothelial cells. Importantly, SMCs just below the internal elastic lamina in the media also express the embryonic phenotype *(double arrow:* note dark staining on some SMCs in the media below the translucent internal elastic lamina; medial SMCs usually express SM1 and SM2 but not SMemb). No mononuclear cells are present. (*Magnification bar* = 10 μm). D to F, Fourteen-day specimens: D, SM1; E: SM2; F, SMemb. D, At 14 days, the intimal lesion above the internal elastic lamina *(white arrowheads)* is seen to be composed primarily of SMCs, all of which express SM1. E, Fewer intimal SMCs express SM2 than SM1; all medial SMCs express SM2 as expected. F, Many intimal SMCs express the embryonic phenotype, SMemb, as well as some medial SMCs just below the internal elastic lamina. (*Magnification bar* = 50 μm.) G to I, Thirty-five–day specimens with changes characteristic of chronic lesions: G, SM1; H, SM2; I, SMemb. Note that the intimal area above the internal elastic lamina *(arrowheads)* occupies the entire photograph. G, Intimal SMCs, expressing SM1, have become spindle shaped. H, These intimal SMCs are no longer expressing SM2. I, The vast majority of intimal SMCs now co-express the embryonic phenotype, SMemb. (*Magnification bar* = 50 μm.)

Discussion

We followed neointimal formation in a new model of rabbit orthotopic carotid arterial allotransplantation to study the phenotypic transformation of vascular SMCs in transplant arteriopathy. Importantly, unlike balloon injury models, the endothelium remains intact in this model. Also validating this model are the close parallels between lesion development in these carotid transplants with the changes seen in coronary arteries in acute and chronic rejection after cardiac transplantation.[22,24,25] Additionally, the orthotopic position

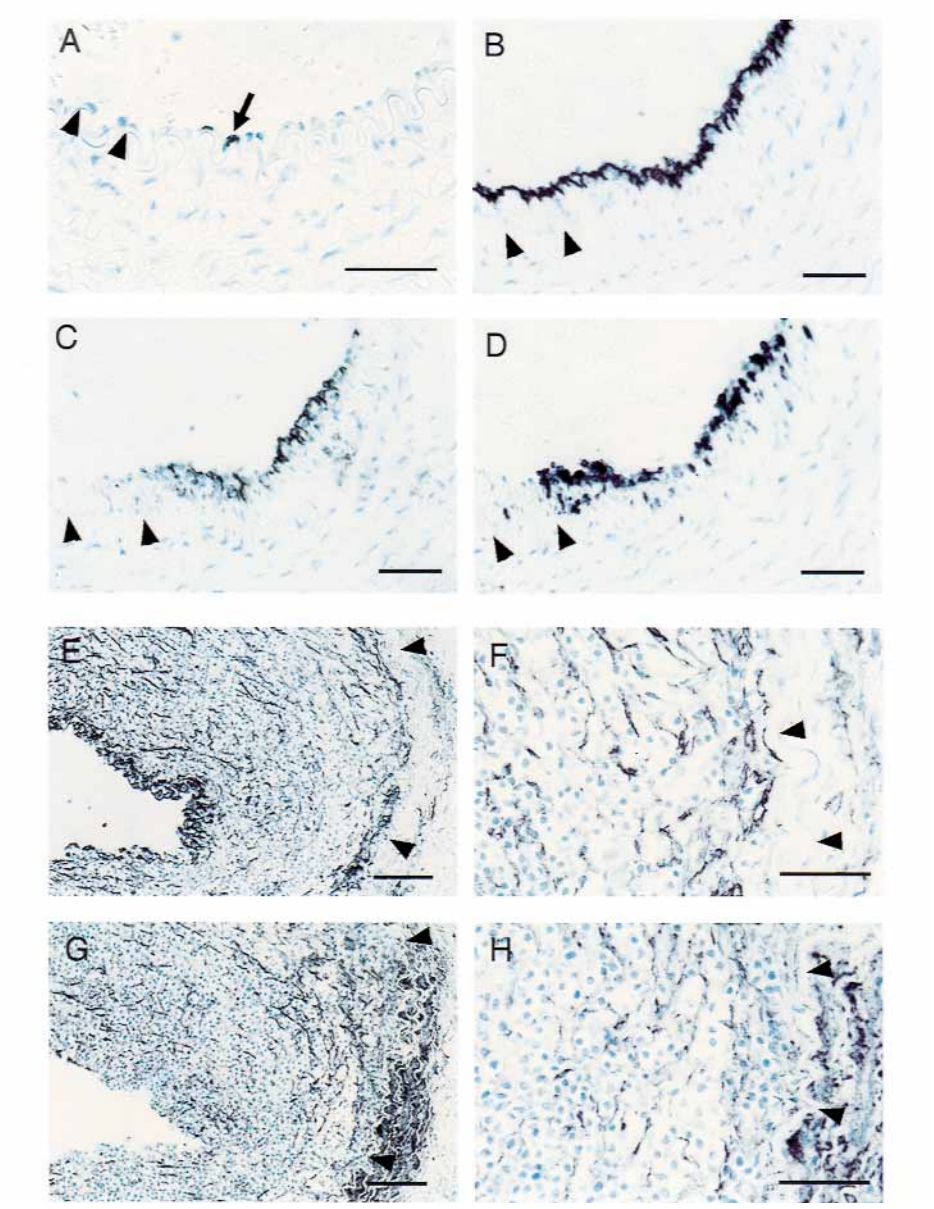

Figure 6. VCAM-1 expression in carotid allografts. In all photographs, the *arrowheads* mark the internal elastic lamina. A, Seven days: VCAM-1 expression (stained in *black,* mAb Rb1/9, methyl green nuclear counterstain) on occasional endothelial cells *(black arrow).* Magnification = 50 μm. B to D, Fourteen days: A specimen with a small intimal lesion. B, Platelet endothelial cell adhesion molecule-1 staining (in *black,* as an endothelial marker) demonstrating an intact endothelium; C, VCAM-1 staining (in *black,* mAb Rb1/9), demonstrating VCAM-1 expression on endothelium; D, T-lymphocytes are stained *black* (mAb Tib 188), demonstrating that endothelial VCAM-1 expression is overlying the area of T-lymphocyte infiltration. *Magnification bar* = 50 μm. E to H, Thirty-five–day specimen with a large intimal lesion, comparing the localization of VCAM-1 expression and SM1 expression on the same specimen. Internal elastic lamina is marked by *arrowheads.* Note that the intima comprises most of the field. Panels E and G are low magnification (*magnification bar* = 100 μm); panels F and H are the same specimens at higher magnification (*magnification bar* = 50 μm). E and F, VCAM-1 expression (in *black,* mAb Rb1/9). VCAM-1 expression is seen both on endothelium and on the elongated SMCs throughout the neointima in a pattern consistent with SMC, not leukocyte, expression. Deep in the neointima, VCAM-1 is also seen in SMCs concentrated just above the internal elastic lamina. G and H, SM1 staining in *black.* SM1 expression defining SMCs in the same 35-day specimens. The pattern of SMC expression in the neointimal SMCs is similar to VCAM-1 expression. Dark SM-1 staining is also seen in the media below the internal elastic lamina as expected (all medial SMCs express SM1).

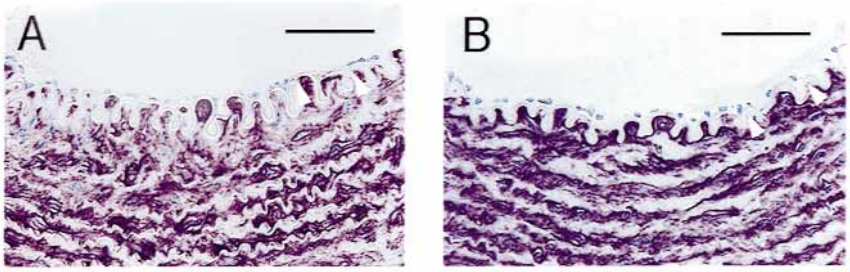

Figure 7. Sham-operated carotid artery specimens stained with antibody to SM1. A, Seven days. B, Thirty-five days. (Methyl green counterstain, *magnification bar* = 50 μm). The internal elastic lamina is visualized as a translucent line *(white arrowheads)* above the heavily SM1-positive SMCs in the media. There is no detectable lesion: that is, no intimal SMCs are identified above the internal elastic lamina.

of the carotid graft ensures that shear forces will not be unnaturally distorted. Most pertinent to the clinical situation, however, is that this model simulates not only the very beginning of transplant arteriopathy but also the transition point between early inflammatory lesions and later chronic lesions composed primarily of SMCs and extracellular matrix, albeit over a condensed time frame compared with human transplant arteriopathy.

SMC Infiltration May Precede Mononuclear Infiltration

We have shown that early SMC infiltration precedes the infiltration of both T-lymphocytes and macrophages in this model of transplant arteriopathy. Early SMC infiltration of coronary vessels was similarly noted by Kuwahara and associates[21] in rabbit cardiac transplants. These findings not only differ from our own expectations of the pathogenesis of an immune-mediated arteriopathy but also differ from the commonly accepted paradigm for plaque development in both transplant arteriopathy and nontransplant atherogenesis in which infiltrating mononuclear cells are considered as primary instigators of early lesion development.[26-28] In this carotid transplant model, we can also demonstrate that medial vascular SMCs just below the internal elastic lamina begin expressing the embryonic myosin heavy chain phenotype up to a week before mononuclear infiltration, a phenomenon also described in neointimal formation in experimental atherosclerosis[15,16] and restenosis.[13,14] This finding strongly suggests that endothelial activation alone, with cytokine or growth factor elaboration and/or signaling through adjacent SMCs or matrix components, can result in medial SMC phenotypic modulation even without direct contact between mononuclear cells and SMCs. In such an immune-related arteriopathy, endothelial activation is presumably the result of allogeneic leukocyte engagement of endothelial surface adhesion receptors, which can initiate signaling pathways in endothelial cells[29] in addition to activating the circulating T-lymphocytes.

That surgically induced ischemia alone might have caused this early intimal SMC infiltration is unlikely because SMC infiltration was not seen in the sham-operated carotid arteries that were exposed to the same surgical manipulation and ischemic times. Additionally, in these experiments, the transplanted carotid arteries with the longer ischemic times did not have more extensive lesions than those with shorter ischemic times. Nonetheless, without a larger sample size or longer ischemic times, it is impossible to rule out that exposure to ischemia might predispose vascular endothelium to subsequent activation or injury by immune mechanisms, effects that could be synergistic.

The question could be raised as to whether these individual cells below the endothelium at 7 days might represent adherence of circulating recipient-derived stem cells. Although recipient origin is certainly a possibility, the fact that medial SMCs just below the internal elastic lamina are also seen to express this embryonic isoform suggests that these medial cells might be phenotypically transformed cells just before migration into the intima. Alternatively, in both the intima and the media, the cells expressing the embryonic phenotype might represent rests of multipotent cells with the potential for SMC dedifferentiation.[13,30]

SMC Phenotypic Switch to Embryonic Isoforms Accelerates With Mononuclear Cell Infiltration

Even though SMC dedifferentiation into the embryonic phenotypes could be seen at all time points, the percentage of SMCs expressing this abnormal phenotype increased significantly over time. The rapid increase correlated with the arrival of mononuclear cells into the lesion and with extracellular matrix elaboration. Interestingly, in this model, macrophage infiltration was not seen until later in lesion development. Unlike the key early role ascribed to macrophages in nontransplant atherosclerosis,[26-28] the late appearance of macrophages in this model suggests that different mechanisms may be operative in transplant versus nontransplant arteriopathy.

TABLE 1. Changes in intimal SMC phenotypes over time

	7 days (n = 5)	14 days (n = 7)	21 days (n = 6)	35 days (n = 5)
SM1 (cells/section)	106 ± 109	135 ± 72	241 ± 162	868 ± 621*
SM2/SM1 (%)	32 ± 17	19 ± 20	11 ± 7	23 ± 23
SMemb/SM1 (%)	NA	NA	53 23	70 ± 38

Total SMCs, denoted by SM1 expression, per section were counted by computerized image analysis for each specimen. The fractions of the total SMC cells expressing the SM2 and the SMemb phenotypes were quantified as percentages of the SM1-expressing area. For the embryonic phenotype, positive cells were present in 7- and 14-day specimens, but the fraction of SMemb-positive SMCs was too small an area for quantification. Mean values plus or minus the standard deviation are given.
*Multiple comparison $P = .05$ versus 7 and 14 days.

VCAM-1 Expression on Endothelium Overlies Areas of Mononuclear Cell Recruitment

VCAM-1 was clearly expressed by endothelial cells before T-lymphocyte infiltration. VCAM-1 expression was also seen to overlie pockets of leukocyte infiltration composed solely of lymphocytes, suggesting, as has been shown in other models, that endothelial VCAM-1 expression is important in the initial recruitment of mononuclear cells into the allograft.[31-33] In this respect, this function of endothelial VCAM-1 expression may mimic its purported early role in human nontransplant atherogenesis.[1-3,34]

Vascular SMC Expression of VCAM-1 and Embryonic Myosin Heavy Chain Isoforms May Signify an Activated Phenotype

However, both in this model of transplant arteriopathy and in our previous studies of human atherosclerotic plaques,[4] it may be SMC expression of VCAM-1, more than endothelial expression, that characterizes the transition to the pathologic state. In cultured human aortic SMCs, VCAM-1 expression appears to be uniquely linked to the differentiation process in SMCs,[35,36] and, furthermore, to be under different transcriptional control depending on the stage of differentiation.[36] Tumor necrosis factor α–induced expression of VCAM-1 occurs preferentially in undifferentiated SMC populations in vitro,[36] supporting our finding of histologic co-localization of VCAM-1 expression with these dedifferentiated myosin phenotypes in transplant arteriopathy. VCAM-1 expression by SMCs might allow direct interactions with T cells and/or macrophages deep within intimal lesions, with other SMCs expressing the VCAM-1 receptor, very late activation-4,[35] or with extracellular matrix ligands.[37]

The potential import of these phenotypic changes is illustrated by previous reports, which found that experimental atherosclerosis develops only in vessels that contain rests of SMCs expressing these embryonic phenotypes.[17,38] In human pathologic conditions, this same embryonic phenotype (nonmuscle myosin B) has been found in the neointima in human atherectomy specimens with restenosis, a proliferative arteriopathy with pathologic similarities to transplant arteriopathy.[39] If this phenotypic modulation is equivalent to activation of vascular SMCs, then expression of this phenotype might mark cells destined for subsequent clonal expansion,[30,40] proliferation, migration, and/or extracellular matrix production.[9,41] Most important, it has recently been shown that strategies that block phenotypic modulation and encourage persistence of stable adult myosin isoforms may prevent lesion progression in nontransplant arteriopathy.[17,42,43] Defining the cell populations subject to dedifferentiation or embryonic rests with potential for clonal expansion and the factors that influence phenotypic modulation may likewise uncover new therapeutic approaches for transplant arteriopathy. Current immunosuppressive therapies, directed at leukocytes, may not be effective at preventing the development of transplant arteriopathy. Instead, therapeutic strategies targeting SMC activation and dedifferentiation, and/or early endothelial activation and signaling, may be necessary for the prevention of transplant arteriopathy.

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